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**pORF5 plasmid protein of *Chlamydia trachomatis* induces MAPK-mediated pro-inflammatory cytokines via TLR2 activation in THP-1 cells**ZHOU Hui<sup>1,2†</sup>, HUANG QiuLin<sup>3†</sup>, LI ZhongYu<sup>1\*</sup>, WU YiMou<sup>1</sup>, XIE XiaoBing<sup>2</sup>,  
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Infection with *Chlamydia trachomatis* induces inflammatory pathologies in the urogenital tract that can lead to infertility and ectopic pregnancy. Pathogenesis of infection has been mostly attributed to excessive cytokine production. However, precise mechanisms on how *C. trachomatis* triggers this production, and which protein(s) stimulate inflammatory cytokines remains unknown. In the present study, the *C. trachomatis* pORF5 protein induced tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ) and interleukin-8 (IL-8) in dose- and time-dependent manners in the THP-1 human monocyte cell line. We found that intracellular p38/mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)/MAPK signaling pathways were required for the induction of TNF- $\alpha$ , IL-1 $\beta$  and IL-8. Blockade of toll-like receptor 2 (TLR2) signaling reduced induction levels of TNF- $\alpha$ , IL-8 and IL-1 $\beta$ . We concluded that the *C. trachomatis* pORF5 protein might contribute to the inflammatory processes associated with chlamydial infections.

***Chlamydia trachomatis*, pORF5 plasmid protein, mitogen-activated protein kinase, proinflammatory cytokines, TLR2**

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*Chlamydia trachomatis* is a pathogenic microorganism responsible for ocular and sexually transmitted infections. Trachoma caused by serovars A, B, Ba and C, is the world's leading cause of infectious blindness [1]. Infections with genital serovars D-K mostly manifest as urethritis, prostatitis or epididymitis in men, and as cervicitis, salpingitis or endometritis in women [2,3]. The L serovars, including L1, L2, L2a and L3, often cause disseminated diseases. Chlamydial genital tract infection may also significantly increase

the risk for HIV infection and cervical carcinoma [4–6]. The majority of chlamydial infections are asymptomatic, therefore infected individuals often do not receive timely medical treatment, and develop a chronic persistent infection leading to severe complications. Therefore, research on chlamydial virulence factors and pathogenic mechanisms is required to improve diagnosis and prevention of chlamydial infections.

Infection with *C. trachomatis* induces a wide array of inflammatory cytokines and chemokines [7–9]. These likely contribute to chlamydia-induced inflammatory pathologies [10]. The pathologies associated with genital infection and

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ocular trachoma are thought to result from injury during an aggressive inflammatory response. However, the precise mechanisms related to how chlamydia triggers such a response, and which protein(s) stimulate inflammatory cytokines remains unclear.

Along with a highly conserved genome of around 1000 kb of chromosomal DNA, all *C. trachomatis* serovars also contain a 7.5 kb cryptic plasmid [11]. This plasmid encodes eight open reading frames, designated pORF1–8. Although the function of the plasmid has not been mapped, evidence has shown that plasmid-deficient chlamydia fail to induce any immune pathology [12]. This would suggest that the plasmid might be associated with virulence. As the only secreted protein encoded by the plasmid, pORF5 possibly contributes to chlamydia-induced pathologies.

The aims of our study were to determine whether pORF5 can activate monocytes to produce pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-8); and determine whether TLR2 and MAPKs are required for pORF5-induced cytokine expression by monocyte cells. A recombinant pORF5 protein was produced and purified, and then human monocyte cells were incubated with pORF5 in the presence or absence of anti-TLR antibodies and specific inhibitors of MAPKs. We evaluated the effects of pORF5 on IL-1 $\beta$ , IL-8 and TNF- $\alpha$  production.

## 1 Materials and methods

### 1.1 Expression and purification of GST-pORF5 fusion protein

A pGEX-6p/pORF5 recombinant plasmid was transformed into *Escherichia coli* XL1-Blue host cells for expression of the glutathione S transferase (GST)-pORF5 fusion protein. Expression was induced with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA), and the fusion protein extracted by lysing *E. coli* in a lysis buffer (1% Triton X-100, 1 mmol L<sup>-1</sup> phenylmethylsulfonyl fluoride, 75 U mL<sup>-1</sup> of aprotinin, 20  $\mu$ mol L<sup>-1</sup> leupeptin, and 1.6  $\mu$ mol L<sup>-1</sup> pepstatin) and subjecting to sonication. After high-speed centrifugation (12000 $\times$ g) to remove debris, the fusion protein was purified with glutathione-conjugated agarose beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), and subjected to cleavage of the GST tag with PreScission protease (Pharmacia) following the manufacturer's instructions. After concentrating with a Centricon YM-10 (Millipore, Billerica, MA) and removing endotoxins with polymyxin B (Sigma, St. Louis, MO), the purified pORF5 protein was ready to use in macrophage stimulation experiments.

### 1.2 Cell culture and simulation

THP-1 cells (TIB-202; ATCC) were cultured in RPMI 1640 (GIBCO BRL, Rockville, MD) supplemented with 10%

fetal bovine serum (FBS; GIBCO BRL) at 37°C/5% CO<sub>2</sub>. For stimulation experiments, THP-1 cells were seeded in 24-well plates at a density of 10<sup>5</sup> cells mL<sup>-1</sup> and cultivated in 1% FBS overnight. The purified pORF5 was added to cultures as stimuli at the concentrations and times indicated for individual experiments. The *E. coli* lipopolysaccharide (LPS; Sigma) was used as a positive control stimulus. In some experiments, cells were pretreated with mouse anti-TLR2 antibody (Abcam, Cambridge, UK), PD98059 (Sigma) and SB203580 (Sigma) for 30 min prior to pORF5 stimulation.

### 1.3 ELISA for cytokine detection

At the end of culture, the cells were lysed by two consecutive cycles of freezing/thawing to yield maximal recovery of total cytokines. This included both intracellular cytokines and those released into the cell culture. The concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 were measured using human ELISA kits (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions. Briefly, 96-well ELISA microplates were coated with a capture antibody, and after blocking with 5% BSA, cytokine samples were added to the coated plates. This was followed with a biotin-conjugated detection antibody. Antibody binding was measured with horseradish peroxidase-conjugated avidin along with a soluble colorimetric substrate. The absorbance was measured at 405 nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) and cytokine concentrations calculated.

### 1.4 SDS-PAGE and Western blotting

Cell samples were harvested and solubilized with 2% sodium dodecyl sulfate (SDS) sample buffer, then loaded onto 12% SDS polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes, and incubated with primary antibodies, including rabbit monoclonal antibodies against phospho-ERK, phospho-JNK and phospho-p38 (Cell Signaling Technology, Beverly, MA), and mouse monoclonal antibodies against JNK, p38, and ERK (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Following incubation with the primary antibody, blots were incubated with a secondary antibody conjugated to horseradish peroxidase (either goat anti-rabbit or goat anti-mouse immunoglobulin G; Jackson Immunologicals, Westgrove, PA) and visualized using an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotech).

### 1.5 Statistical analysis

Data from three independent experiments are presented as the mean $\pm$ standard deviation. Statistical software (SPSS-13.0) was used to analyze results with Student's *t*-test followed by one-way analysis of variance (ANOVA). A *P*-

value less than 0.05 was considered statistically significant.

## 2 Results

### 2.1 Expression and purification of pORF5 protein

The GST-pORF5 fusion protein was expressed in a prokaryotic expression system and purified with glutathione agarose beads. The purified GST-pORF5 was cleaved with PreScission protease to remove the GST tag. We observed the tagged and purified pORF5 (54 kD), along with the cleaved pORF5 (28 kD) on SDS-PAGE gels (Figure 1A). This result was confirmed by Western blotting analysis using the specific monoclonal mouse anti-pORF5 antibody (clone 2H4) (Figure 1B).

### 2.2 pORF5 protein induces inflammatory cytokines in THP-1 cells

The purified pORF5 induced TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production in THP-1 cells in a dose-dependent manner, for the concentration range 1–8  $\mu\text{g mL}^{-1}$  (Figure 2A). From 1–6  $\mu\text{g mL}^{-1}$ , a correlation between dose and cytokine production was observed. Low concentrations of pORF5 induced low levels of cytokine secretion, while higher concentrations induced higher levels of cytokine production. When the concentration was greater than 6  $\mu\text{g mL}^{-1}$ , production of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 decreased. Cytokine production was also time-dependent from 6–36 h, with TNF- $\alpha$  levels peaking at 6 h post-stimulation. The peak amounts of IL-1 $\beta$  and IL-8 occurred at 12 h post-stimulation (Figure 2B). In all experiments, *E. coli* LPS was used as the positive control, as this is the most effective stimuli in triggering cytokine production. The negative control, phosphate-buffered saline (PBS), barely induced any expression of cytokines.

No endotoxins were detected using a Limulus amoebocyte lysate assay for the purified pORF5 protein. The possibility

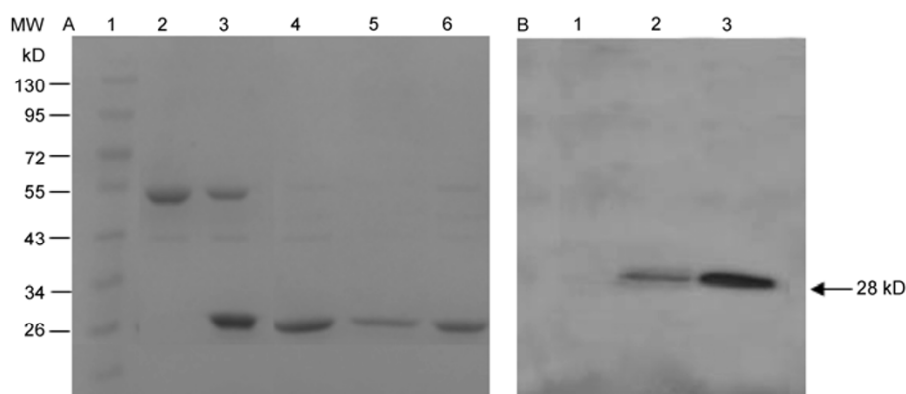
that its pro-inflammatory activity was attributed to LPS contamination was further investigated by testing pORF5 sensitivity to polymyxin B. As shown in Figure 2C, pORF5 pre-treated with 50  $\mu\text{g mL}^{-1}$  polymyxin B had no effect on TNF- $\alpha$  production ( $P>0.05$ ) while blocking most of the LPS-mediated stimulation activity ( $P<0.01$ ).

### 2.3 pORF5 activates ERK and p38/MAPK signaling pathways

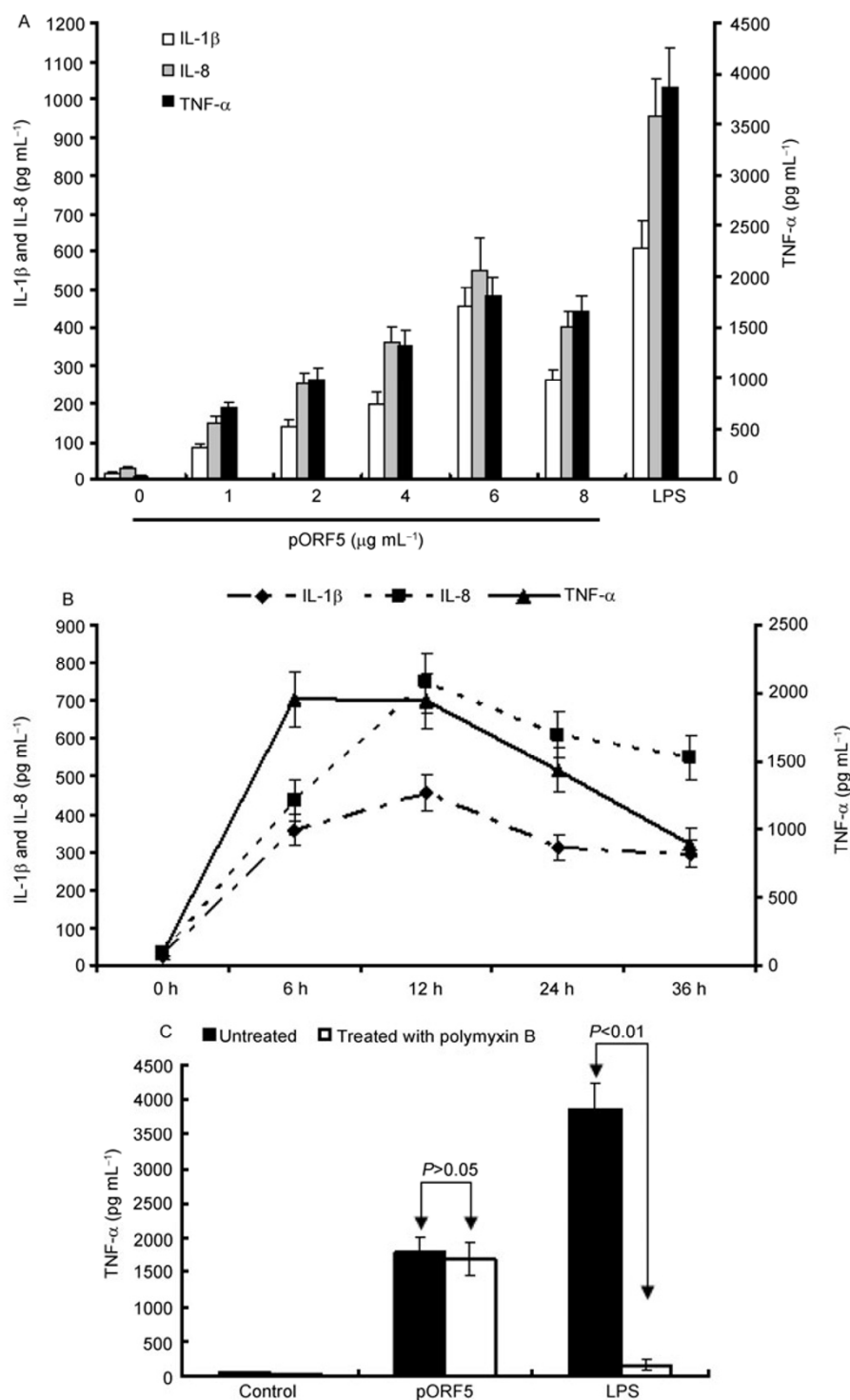
As shown in Figure 3, pORF5 activated p38/MAPK and ERK/MAPK pathways, but was unable to activate the JNK/MAPK. The p38/MAPK and ERK/MAPK pathways were activated at about 15 min after pORF5 stimulation, peaking at 15 and 30 min, respectively, then gradually decreased.

### 2.4 p38/MAPK and ERK/MAPK pathways are involved in pro-inflammatory cytokine production induced by pORF5 protein

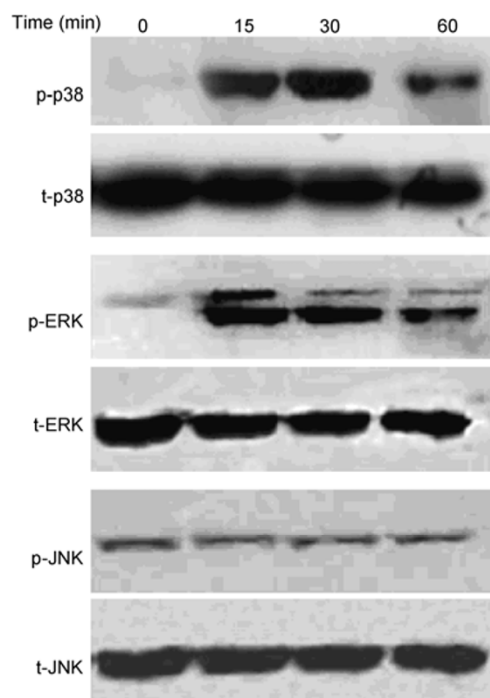
After pretreating with SB202190, a p38/MAPK inhibitor, and PD98059, an ERK/MAPK inhibitor, at three different doses (1, 10 and 30  $\mu\text{mol L}^{-1}$ ) for 30 min, THP-1 cells were stimulated with 6  $\mu\text{g mL}^{-1}$  pORF5 for 12 h. We then examined the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 to assess the effects on the production of different cytokines. As shown in Figure 4, inhibition of p38/MAPK and ERK/MAPK pathways reduced pORF5-induced TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production levels. SB202190 and PD98059 effected pro-inflammatory cytokine production in a dose-dependent manner. Compared with the control group, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 were reduced to 22.4%, 27.6% and 22.8% respectively, when 30  $\mu\text{mol L}^{-1}$  SB202190 was used (Figure 4A). When 30  $\mu\text{mol L}^{-1}$  PD98059 was used, TNF- $\alpha$ , IL-1 $\beta$  and IL-8 levels were reduced by 60.3%, 58.8% and 45.7% (Figure 4B).



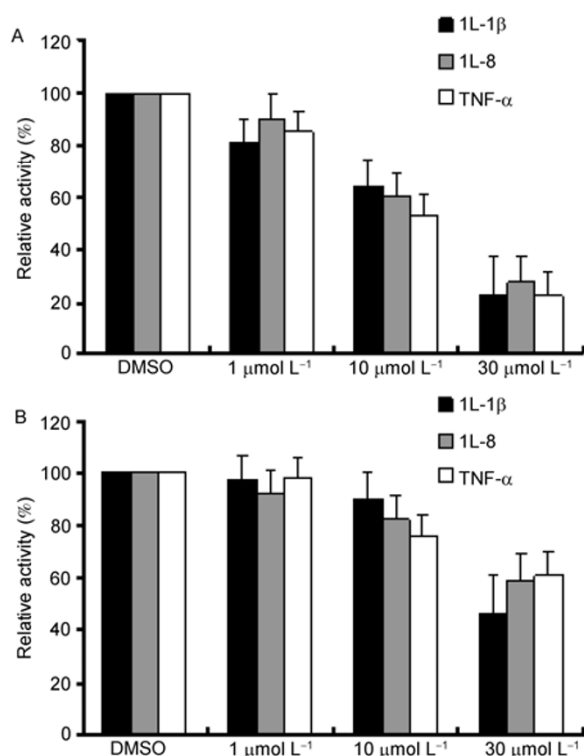
**Figure 1** Purification and identification of the pORF5 protein. A, Coomassie blue-stained acrylamide gel after SDS-PAGE. Lane 1, marker; 2, GST-pORF5; 3, incompletely cleaved GST-pORF5; 4, pORF5 eluate 1; 5, pORF5 eluate 2; 6, pORF5 eluate 3. B, Western blotting assay. Lane 1, XL1-Blue *E. coli* lysate; 2, eluted GST-pORF5 agarose beads after protease cleavage; 3, supernatant of GST-pORF5 agarose beads after cleavage with protease.



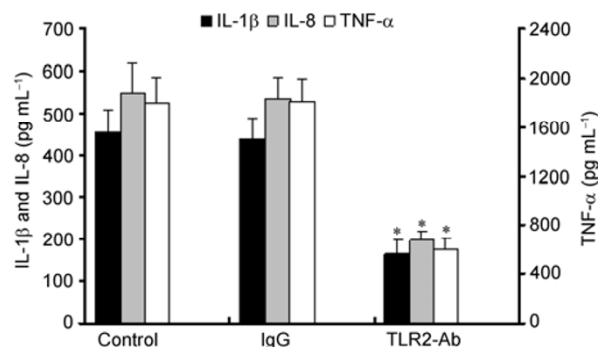
**Figure 2** Induction of inflammatory cytokines by the pORF5 protein. A, Dose-dependent effects of purified pORF5 on the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in THP-1 cells. THP-1 cells were incubated for 12 h with or without pORF5 at final concentrations ranging from 2–8  $\mu\text{g mL}^{-1}$ . LPS at a final concentration of 100  $\text{ng mL}^{-1}$  was used as a positive control, and PBS was used as a negative control. Samples were collected and assayed for TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production. Data were obtained from three independent experiments. The error bars indicated standard deviations. B, Time-dependent effects of purified pORF5 on TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production in THP-1 cells. THP-1 cells were incubated with 6  $\mu\text{g mL}^{-1}$  pORF5 for 0–36 h. Samples were harvested and assayed for TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production. Data are expressed as mean $\pm$ SD ( $n=3$ ). Error bars indicate standard deviations. C, Effects of polymyxin B on pORF5- and LPS-induced TNF- $\alpha$  production in THP-1 cells. pORF5 or LPS was pre-treated with 50  $\mu\text{g mL}^{-1}$  polymyxin B for 1 h before stimulating THP-1 cells. pORF5 (6  $\mu\text{g mL}^{-1}$ ) or LPS (100  $\text{ng mL}^{-1}$ ) were then added to culture medium. After a 12-h incubation, samples were harvested and the concentration of TNF- $\alpha$  determined by ELISA. The data were from three independent experiments. Error bars indicate standard deviations.



**Figure 3** Activation of MAPKs induced by pORF5 in THP-1 cells. THP-1 cells were incubated with  $6 \mu\text{g mL}^{-1}$  pORF5 for 0–60 min. Activation of p38, ERK and JNK was detected by Western blotting using the appropriate specific antibodies.



**Figure 4** Effects of specific MAPK inhibitors on pORF5-induced cytokine production. THP-1 cells were pre-incubated with three different concentrations of SB202190 (A), PD98059 (B) or dimethyl sulfoxide (DMSO) for 30 min. We then added  $6 \mu\text{g mL}^{-1}$  pORF5 to the medium. TNF- $\alpha$ , IL-1 $\beta$  and IL-8 levels were detected by ELISA. Our results were representative of three independent experiments.



**Figure 5** TLR2 is involved in the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-8. THP-1 cells were pre-incubated with  $10 \mu\text{g mL}^{-1}$  anti-TLR2, or isotype-matched mouse IgG for 30 min. Purified pORF5 was added at a final concentration of  $6.0 \mu\text{g mL}^{-1}$ . TNF- $\alpha$ , IL-1 $\beta$  and IL-8 concentrations were determined by ELISA. \*,  $P < 0.01$  compared with the control groups.

## 2.5 TLR2 is involved in pORF5-induced proinflammatory cytokine production

Compared with the control group, blocking TLR2 with a neutralizing anti-human TLR2 antibody resulted in significant reduction of pro-inflammatory cytokine production induced by pORF5 ( $P < 0.01$ ). The TLR2 antibody had negligible effect on LPS-inducing cytokine production (data not shown). The control IgG did not influence the production of IL-1 $\beta$ , IL-8 and TNF- $\alpha$  (Figure 5).

## 3 Discussion

*C. trachomatis* is an obligate intracellular bacterial pathogen. During intravacuolar growth, *C. trachomatis* must secrete factors into the inclusion membrane [13–15] or the host cell cytosol [16–18] for interacting with and/or manipulating host cell signaling pathways [19–21]. As a result of chlamydia-host interactions, host cell genes encoding inflammatory cytokines and chemokines are often activated. Previous studies have been shown that a wide array of inflammatory cytokines and chemokines, including IL-1 $\alpha$ , IL-6, IL-8, IL-18, TNF- $\alpha$  [7–9], are produced *in vitro* and/or *in vivo*. These are believed to contribute to chlamydia-induced pathologies [10].

There are 19 different serovars among *C. trachomatis* species. Almost all serovars contain the 7.5 kb cryptic plasmid [11]. Loss of this plasmid has been associated with immunopathology and infectivity [22,23]. Vaginal inoculation of mice with a plasmid-cured derivative of *C. muridarum* reduced bacterial burden almost 10-fold, and there was an associated absence of oviduct pathology [12]. Other research has also indicated that plasmid-negative strains had significantly higher 50% infective dose values relative to a wild-type matched isolate [24]. These results indicate that this cryptic plasmid might be associated with virulence attributes. Of the eight plasmid-encoded proteins, pORF5 has

been shown to be the only protein that is secreted and distributed in the cytosol of infected cells. The remaining seven proteins localize inside chlamydial inclusions [17]. Since *Chlamydia* restricts its own biosynthetic activity within an inclusion, pORF5 might be relevant to chlamydial interactions with host cells. Our findings show that purified pORF5 induced TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production in dose- and time-dependent manners. The inflammatory reactions peaked when the pORF5 concentration was 6  $\mu\text{g mL}^{-1}$ . Higher concentrations of pORF5 were inhibitory to cytokine production, which may indicate some interference with the biological function of the cells. TNF- $\alpha$  peaked at 6 h post-stimulation, while IL-1 $\beta$  and IL-8 production occurred 12 h after stimulation. Our observations clearly show pORF5 induces cytokine production, and suggest that pORF5 might contribute to chlamydia-induced inflammatory pathologies.

MAPKs comprise a family of proline-directed serine/threonine kinases. These are important signaling pathways for the host immune response. Three major MAPKs, ERK, p38 and JNK have been identified in the intracellular network that transduces extracellular signals to an intracellular response. Previous studies have demonstrated that MAPKs contribute to the production of different cytokines during chlamydial infection [25,26]. We hypothesized that MAPKs were also involved in the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 induced by pORF5. This hypothesis was verified by our results derived from the phosphorylation detection and specific inhibitor studies. The effects of specific inhibitors on cytokine production varied. When p38/MAPK was blocked 30  $\mu\text{mol L}^{-1}$  SB202190, levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 were reduced. Blocking ERK/MAPK with 30  $\mu\text{mol L}^{-1}$  PD98059 further reduced TNF- $\alpha$ , IL-1 $\beta$  and IL-8 levels. The p38/MAPK pathway appeared to be more sensitive to cytokine responses following pORF5 stimulation.

TLRs are transmembrane receptors within the IL-1 receptor superfamily. They are regarded as the major molecules by which hosts recognize invading microorganisms. Recent experiments have shown that TLR2 plays a fundamental role in chlamydia pathology. TLR2-deficient mice did not develop oviduct pathology after infection, demonstrating that TLR2 signaling is directly involved in disease development [27,28]. Data from mouse models have demonstrated that plasmid-deficient *C. muridarum* failed to activate TLR2-dependent immune responses *in vivo* or *in vitro* [29]. These observations suggest that the plasmid regulates TLR2-dependent signaling and participates in chlamydial pathogenesis. We found that an anti-TLR2 antibody significantly inhibited inflammatory cytokine production. This indicates that TLR2 likely participates in the production of inflammatory cytokines induced by pORF5.

In summary, we have demonstrated that pORF5 induces expression of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in THP-1 cells. The induction of these cytokines has subsequent effects upon TLR2, p38/MAPK and ERK/

MAPK. However, further research upstream and downstream of the MAPK pathways, and the transcription factors need to be conducted. Our findings presented herein now provide a better understanding of cytokine release and the pathogenicity of *C. trachomatis* at a molecular level.

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